THE HYDROPHOBIC EFFECT: FORMATION OF MICELLES AND BIOLOGICAL MEMBRANES

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the constant term. It is seen that the increment per CH_2 group is the same as that in equation 3-2, even though the organic solvent in this case is not a pure hydrocarbon. This agreement may be attributed to the probable hydrogen bonding between OH groups in a liquid alcohol, which would leave the distal portions of hydrocarbon tails essentially in a hydrocarbon environment. (See discussion of solubilities of hydrocarbons in alcohols in Chapter 2.) The constant term in equation 3-3 is very much smaller than that for carboxylic acids, given in equation 3-2, indicating that the terminal OH group is less hydrophilic than a COOH group, which is not surprising, since the latter can form a larger number of hydrogen bonds. Taking the contribution of the terminal methyl group to $\mu^{\circ}_{ROH} - \mu^{\circ}_{W}$ to be -2100 cal/mole, as before, we find that the contribution of the terminal OH group becomes $+1340$ cal/mole if the CH_2 group proximal to the OH group is assumed to make no contribution to $\mu^{\circ}_{ROH} - \mu^{\circ}_{W}$.

These results support the idea expressed at the beginning of this chapter, that the contributions of the hydrophilic and hydrophobic portions of an amphiphile to the free energy of interaction with solvent should be nearly independent. One might have expected the increment per carbon atom in equation 3-2 to be a few percent larger, that is, the same as in equations 2-3, 2-5, and 2-6. However, it must be recognized that we are dealing with isolated experimental studies and that the absolute accuracy of the measurements may not be as good as the self-consistency expressed by the close adherence to the linear relations. A somewhat smaller increment per carbon atom in equation 3-3 is theoretically not unexpected because the organic solvents in this case are a series of alcohols.

Reference should be made to a study by Schrier and Schrier (1967), which indicates that the effect of added salt on μ°_{W} of organic amides is also an additive function of approximately independent contributions from polar and nonpolar groups.

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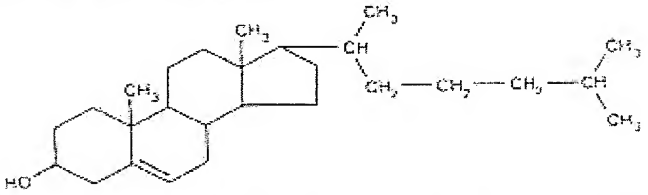
TWELVE

BIOLOGICAL LIPIDS

The biochemist uses the term "lipid" to define all organic molecules of biological origin that are highly soluble in organic solvents and only sparingly soluble in water. Included in this definition are fats, some hormones and vitamins, and many other substances of diverse chemical identity and biological function. The discussion in this chapter will be confined to lipids present in biological membranes or in soluble lipoprotein complexes, and they fall into two classes:

1. Amphiphile molecules or ions of the type discussed previously in this book, containing a strongly hydrophilic head group and one or more (most often *two*) long hydrocarbon tails.

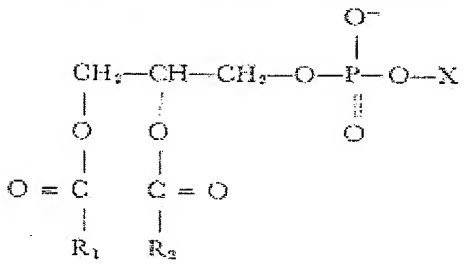
2. Cholesterol and acyl esters of cholesterol. These substances do not resemble any we have previously considered: their principal constituent is pure hydrocarbon in the form of the relatively rigid steroid ring. Cholesterol, the structural formula for which is



may be considered amphiphilic by virtue of possession of a single OH group. In cholesteryl esters this OH group forms an ester link with a fatty acid. In either case the affinity for water is very weak compared to that of the strongly hydrophilic head groups of other membrane lipids.

The most abundant lipids in the first category contain two hydrocarbon chains attached to a single polar head group. Among lipids of this type are

most of the phosphoglycerides, which have the general formula



(I)

where R_1 and R_2 are hydrocarbon chains. The head group bears a net negative charge at neutral pH if the X group in this formula is neutral or zwitterionic, as follows:

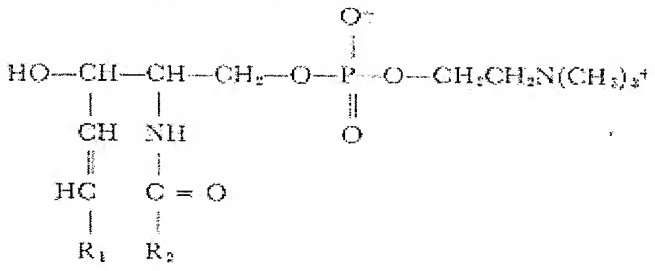
Phosphatidic acid	$-X = -H$
Phosphatidylserine	$-X = -CH_2CH(NH_3^+)COO^-$
Phosphatidylinositol	$-X = -C_6H_6(OH)_5$
Phosphatidylglycerol	$-X = -CH_2CHOHCH_2OH$

Alternatively the head group may be zwitterionic at neutral pH, if the X group bears a positive charge, as in

Phosphatidylethanolamine	$-X = -CH_2CH_2NH_3^+$
Phosphatidylcholine	$-X = -CH_2CH_2N(CH_3)_3^+$

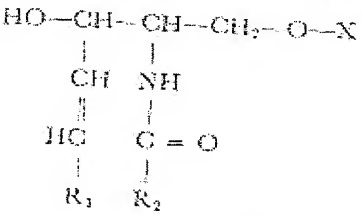
Aminoacyl derivatives of phosphatidyl glycerol occur in some bacteria (Houtsmuller and Van Deenen, 1965; Lennarz, 1972). Included in this category is the lysyl ester of phosphatidylglycerol, which bears two positive charges, conferring a net positive charge on the head group as a whole. Apart from this instance, phosphoglycerides with positively charged head groups are rare or nonexistent.

Plasmalogens represent a variant form of I in which one of the ester links is replaced by an ether linkage, and similar molecules with two ether linkages have also been observed. Another lipid, chemically quite distinct but geometrically similar, is sphingomyelin



(II)

Most glycolipids are also sphingolipids, with the general formula



(III)

The carbohydrate moiety X may be a single neutral hexose sugar (as in cerebrosides), a sulfated sugar (cerebroside sulfate or sulfatide), or a more complex oligosaccharide. The head group of sulfatides bears a negative charge, as does the head group of gangliosides, in which X is a complex oligosaccharide containing one or more moles of sialic acid.

Mitochondria contain considerable quantities of cardiolipin, in which *four* hydrocarbon chains are attached to a single head group. This lipid is essentially a dimeric form of I. Two molecules of the diacyl glycerophosphate moiety are linked by one molecule of glycerol (i.e., —X is replaced by $-CH_2CHOHCH_2-$). There are two negative charges on the head group.

Lipids with a single hydrocarbon chain per head group occur in only very small quantities.[1] Lipids in this category are the lysophospholipids (formula I with one of the acyl group removed) and the free fatty acid anions. As noted in Chapter 10, all hydrophobic or amphiphilic molecules tend to be incorporated in micelles formed by other amphiphiles and, considering membranes tentatively as having some resemblance to micelles formed by the principal membrane lipids, the presence of some minor components, such as fatty acid anions, may simply be a reflection of their presence in the medium with which the membrane is in contact.

Analytical data for the lipid content of several membranes are shown in Table 12-1. They are intended primarily to show that composition with respect to lipid classes is quite different for different membranes. The common features of all the data are (1) that 75% or more of membrane lipid consists of amphiphilic molecules with two hydrocarbon chains per head group, (2) that about 20 to 30% of the lipid contains anionic head groups, and (3) that there are no cationic head groups at all, those that are not anionic being neutral or zwitterionic. (As noted above, positively charged head groups do occur in some bacterial membranes.)

[1] The membrane of the hormone-secreting granules of the adrenal cortex may constitute an exception; it has been shown to contain about 15% of lysophosphatidylcholine (Winkler and Smith, 1968). A similar high content of lysophosphatidylcholine in zymogen-secreting granules of the pancreas has however been shown to be an artifact, arising from enzymatic degradation during the isolation procedure (Meldolesi et al., 1970).

Table 12-1. Lipid Compositions of Some Biological Membranes[a] (expressed as percent by weight of total lipid)

	Human Erythrocyte[d]	Human Myelin[e]	Beef Heart Mitochondria[f]	E. Coli[g]
Phosphatidic acid	1.5	0.5	0	0
Phosphatidylcholine[b]	19	10	39	0
Phosphatidylethanolamine[b]	18	20	27	65
Phosphatidylglycerol	0	0	0	18
Phosphatidylinositol	1	1	7	0
Phosphatidylserine	8.5	8.5	0.5	0
Cardiolipin	0	0	22.5	12
Sphingomyelin	17.5	8.5	0	0
Glycolipids	10[h]	26[i]	0	0
Cholesterol[c]	25	26	3	0

[a] Quantitative lipid analysis is a rapidly developing science, and many of these figures are likely to change as it progresses. In bacterial membranes, lipid composition depends to some extent on growth phase and nutritional factors.

[b] Up to one-third of the phosphatidyl derivatives may be in the plasmalogen form, that is, with an ether rather than an ester link between the hydrocarbon chain and the glyceryl moiety. For our purposes the distinction is not important.

[c] The figures are for free cholesterol. Cholesterol esters occur in soluble lipoproteins, but have not been found in membranes.

[d] Rouser et al. (1968).

[e] Dickerson (1968), Rouser et al. (1968). The values given are for central nervous system myelin.

[f] Rouser et al. (1968). The values given are for total mitochondrial lipid. Inner and outer membranes differ significantly in composition.

[g] Ames (1968).

[h] 80% of the glycolipid is cerebroside, 20% is sulfatide.

[i] Principally polyhexosides and gangliosides.

The hydrocarbon chains of biological lipids are invariably very long. Only small amounts of fatty acids with fewer than 16 carbon atoms are usually obtained upon hydrolysis of diacyl lipids, and chain lengths to 24 carbon atoms occur frequently. An almost universal feature is the presence of unsaturated fatty acids,[a] typically to the extent of about 50% of the total

[a] In some bacterial lipids a methylene group may be added across the unsaturated bond to produce a cyclopropyl group, or branched saturated fatty acids may under certain conditions replace unsaturated fatty acids. The important factor is geometrical and not chemical: about half the acyl chains must have structures that prevent them from forming parallel close-packed arrays with linear saturated hydrocarbon chains.

fatty acid content, although there are exceptions. For example, membranes of lung tissues contain hydrocarbon chains derived predominantly from saturated fatty acids, dipalmitoyl phosphatidylcholine being the principal constituent (Abrams, 1966). The fatty acid composition of the microorganism *Mycoplasma laidlawii B* can be altered dramatically by regulation of the growth medium, and the organism is viable with a saturated fatty acid content of as much as 90% (McElhaney and Tourtellotte, 1969). In the normal situation, where the number of saturated and unsaturated hydrocarbon chains is about equal, one chain of each kind is often present on the same molecule: in the phosphoglycerides (formula I) of many species, R_1 tends to be a saturated fatty acid and R_2 an unsaturated fatty acid. Unsaturated fatty acids may contain up to six double bonds. Double bonds in unsaturated fatty acids from bacterial or animal membranes are usually in the *cis*-configuration and, in polyunsaturated acids, tend not to be conjugated. Reviews by Rouser et al. (1968) and Hill and Lands (1970) may be consulted for additional information.

All of the biological lipids described above, except cholesterol and its esters, resemble the simpler amphiphiles discussed in preceding chapters. They are expected to form micelles in aqueous media and, because their constituent hydrocarbon chains are invariably very long, the concentration of unassociated lipid in equilibrium with the micelles (which we shall continue to assume is essentially the same as the critical micelle concentration) should be very small. For the predominant type of molecule, containing two hydrocarbon chains per head group, the micelle is expected to be of the bilayer type, and there should be a tendency for the bilayers to form closed vesicles (Chapter 9). Mixtures of lipids should readily form mixed micelles and, if most of the constituent molecules contain two hydrocarbon chains per head group, the mixed micelles should also be bilayers. All available experimental data, some of which will be summarized in this chapter, support these predictions.

In considering the physicochemical data to be presented we should keep in mind the likelihood, as discussed in Chapter 6, that micellar structures formed by amphiphile molecules with very long *saturated* hydrocarbon chains may have hydrophobic cores in which the hydrocarbon chains are in an ordered paracrystalline array at room temperature, instead of being liquid. For molecules with *unsaturated* hydrocarbon chains, on the other hand, the melting point for ordered structures is expected to be below room temperature, and the hydrophobic cores may accordingly be in a fluid state. The data to be presented in this and the following chapter will in fact demonstrate that ordered and disordered hydrophobic cores exist much as expected, and the results of the following chapter will permit us to refine our concepts of the meaning of "order" and "disorder" in these systems.

Many of the results to be discussed were obtained with lipids isolated from natural sources, containing molecules with a mixture of hydrocarbon chains. The material of this type that has been employed more frequently than any other is egg yolk phosphatidylcholine. This material is pure with regard to the head group, but contains a mixture of acyl chains, about 50% of them saturated and 50% unsaturated. Principal constituents after hydrolysis of the ester linkage were found in a typical analysis (Huang et al., 1964) to be palmitic acid, stearic acid, oleic acid (one double bond) and linoleic acid (two double bonds). A small amount of $C_{19}H_{31}COOH$ (four double bonds) was also present. A typical individual molecule in the mixture probably contains one saturated and one unsaturated hydrocarbon chain, as mentioned earlier. Ordered arrays of the hydrocarbon chains have low stability in a mixture of this kind, and egg phosphatidylcholine will be seen to resemble pure synthetic molecules with unsaturated hydrocarbon chains in that it forms bilayers with a liquid hydrophobic core at room temperature. In terms of a number of physical properties not discussed here, the egg yolk product resembles pure dioleyl phosphatidylcholine (DeGier et al., 1968).

CRITICAL MICELLE CONCENTRATION

Because the cmc values are so small, they are difficult to measure. Experimental values are available for palmitoyl lysophosphatidylcholine, which contains only one hydrocarbon chain, and for dipalmitoyl phosphatidylcholine. They are shown in Table 12-2. This table also shows predicted values, based on extrapolation of the data shown in Figs. 7-2 and 7-3. Since no data are available for simple amphiphiles containing the phosphatidylcholine head group, we have used the results for the N-alkyl betaines which, like the phosphatidylcholines, contain a zwitterionic head group, as a suitable model. Predicted values for the cmc would have been lower by a factor of 10 if the alkyl glucosides had been used as a basis for the extrapolation. For the dipalmitoyl derivative we have assumed that the second hydrocarbon chain exerts an effect equivalent to about 60% of that which would result from extending a single chain by the same number of carbon atoms, as Fig. 7-3 suggests. This leads to the prediction that the cmc of dipalmitoyl phosphatidylcholine should be about the same as the cmc of the corresponding lysophosphatidylcholine with a C_{24} saturated hydrocarbon chain. The predictions made in this way are seen to be in excellent agreement with the experimental results, which suggests that the cmc of most pure lipids of this type can probably be estimated reasonably well on the basis of the results for simpler amphiphiles.

Table 12-2. Critical Micelle Concentrations of Biological Lipids in Aqueous Solution at 25°C

	cmc (moles/liter)	cmc (mole fraction)	$\bar{\mu}^{\circ}_{micelle} - \bar{\mu}^{\circ}_{ir}$ (kcal/mole)[a]
Palmitoyl[b] *Lysophosphatidylcholine*			
Observed[c]	$< 12 \times 10^{-3}$	$< 2.1 \times 10^{-5}$	< -7.7
Calculated[d]	6×10^{-4}	1.0×10^{-6}	—
Dipalmitoyl[b] *Phosphatidylcholine*			
Observed[e]	4.7×10^{-10}	8.4×10^{-12}	-15.1
Calculated[d]	11×10^{-10}	21×10^{-12}	—

[a] Using equation 7-3 with $f_{\infty} = 1$.

[b] The palmitoyl moiety contains a C_{16} hydrocarbon chain.

[c] Lewis and Gottlieb (1971). Robinson and Saunders (1958) observed a cmc in the range of 2 to 20 $\times 10^{-5}$ *M* for a lysophosphatidylcholine with mixed hydrocarbon chains.

[d] Based on extrapolation of the data of Figs. 7-2 and 7-3, by using the betaine head group as representative of the phosphatidylcholine head group. See text for details.

[e] Smith and Tanford (1972).

The possibility that the hydrocarbon chains of dipalmitoyl phosphatidylcholine may actually be in an ordered paracrystalline array instead of in a liquid core should not have a large effect on the cmc. The free energy of melting of the ordered array is probably less than 1 kcal/mole and the difference between the cmc of otherwise identical micelles with ordered and liquid cores should thus be less than a factor of 5. This difference is not much greater than the precision with which the cmc can be measured: the probable error is about a factor of two.

SOLUBLE MICELLES

The micelles formed by lysophosphatidylcholines are small and globular, similar to those observed for simpler single-chain amphiphiles. The value for $\bar{n}$ is about 180, both for synthetic palmitoyl lysophosphatidylcholine (Lewis and Gottlieb, 1971) and for a mixed product obtained by enzymatic cleavage of egg yolk phosphatidylcholine (Saunders, 1966). The intrinsic viscosity of the latter was found to be 4 cc/g. Because of the virtual absence of phospholipids with a single hydrocarbon chain in biological membranes, micelles of this type are not of much interest, and little work has been done on them.

Phospholipid molecules containing two hydrocarbon chains are relatively difficult to disperse in aqueous solution in micellar form. When water is added to dry phospholipid, swelling takes place, with formation of liquid crystalline phases of different kinds (see below). Further addition of water tends to lead to dispersal of this lipid without disruption of the ordered aggregated structures. This is probably due to the tendency of bilayers to form closed structures in the presence of water. Electron micrographs of phospholipid preparations containing water or of discrete "liposomes" obtained from them (Dervichian, 1964; Bangham et al., 1965) invariably show multilayered closed structures, such as illustrated by Fig. 12-1, which cannot be further subdivided without rupture of the individual layers.

As was first observed by Saunders et al. (1962) small soluble micelles can be obtained from multilayered structures by ultrasonic irradiation. The micelles so formed represent a mixture of vesicles, some still bounded by several bilayers. A fraction containing only vesicles bounded by a single bilayer can however be separated from such a mixture by gel exclusion chromatography. A detailed study of such vesicles, derived from egg yolk phosphatidylcholine, has been reported by Huang (1969). They were found to be quite uniform in size, with a molecular weight of 2.1×10^6, corresponding to $\bar{m} = 2680$. Electron micrographs, the Stokes radius as measured by gel chromatography and an intrinsic viscosity of 4.1 cc/g are all consistent with a roughly spherical vesicle, with an external diameter of about 250 Å and an internal cavity with a diameter of about 140 Å containing about 0.6 g solvent (mostly water) per g of phospholipid. Both the thickness of the bilayer and the area occupied per head group in these vesicles are consistent with results obtained from X-ray diffraction of liquid crystalline egg phosphatidylcholine (see below).

Similar vesicles, bounded by a single bilayer, but less uniform and of somewhat larger average size, have been obtained from soy bean lipids (Miyamoto and Stoeckenius, 1971), as illustrated by Fig. 12-1*b*. Clear dispersions can be obtained by ultrasonic irradiation from a variety of lipids, and it is probable that they contain vesicles of the same general type. It is probable that any lipid that preferentially adopts the bilayer structure can form such vesicles provided that it contains some unsaturated hydrocarbon chains. There is one report (Saunders, 1966) that they cannot be obtained at room temperature from phosphatidylcholines with identical long saturated fatty acid chains, which would imply resistance to the imposition of curvature on the bilayer because of the tendency to form ordered arrays of the fatty acid chains. More recent work (Sheetz and Chan, 1972), however, has shown that dipalmitoyl phosphatidylcholine does form small vesicles on prolonged sonication, with partial retention of an ordered arrangement of acyl chains.

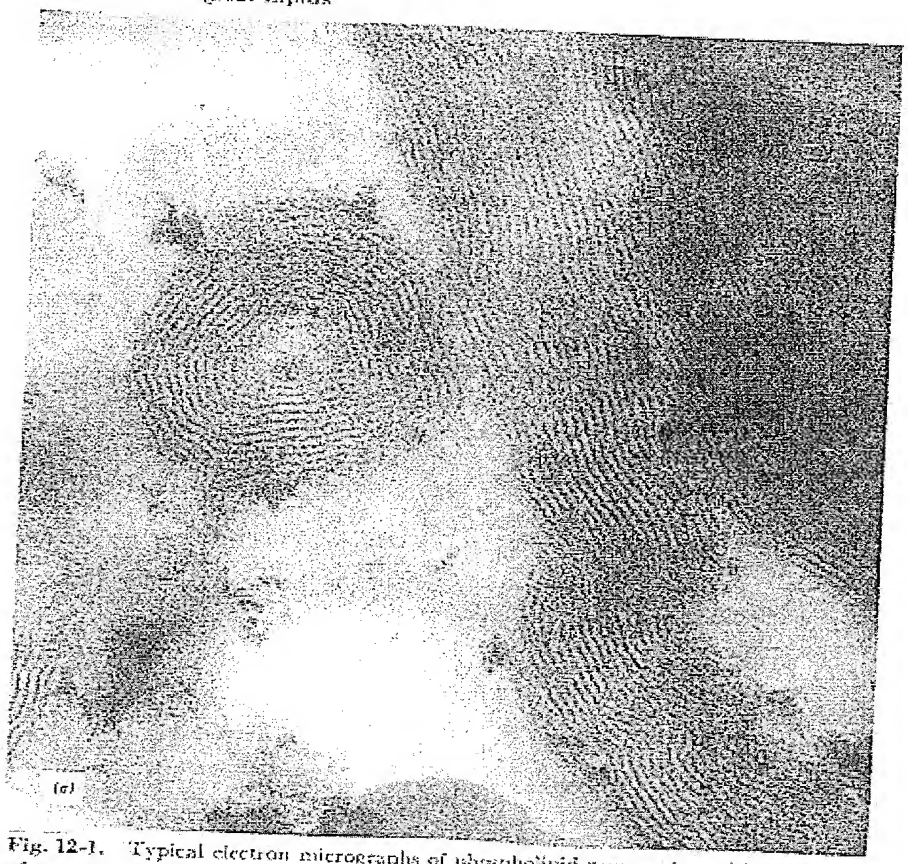


Fig. 12-1. Typical electron micrographs of phospholipid preparations: (*a*) in the form of extended bilayers and multiwalled vesicles (reprinted with permission of Pergamon Press, Ltd., from Dervichian, 1964);

LIQUID CRYSTALLINE PHASES AT HIGH LIPID CONCENTRATIONS

As was true for simple amphiphiles (Chapter 9) biological lipids form a variety of ordered phases (liquid crystals) in mixtures with water when the lipid content is high. As previously noted, these structures tend to persist even when large amounts of water are added, unless disrupted by sonication. In the case of simple amphiphiles the molecular arrangement within the micellar aggregates is greatly altered as the water content is reduced, but for lipids with two hydrocarbon chains per molecule the bilayer arrangement is a common feature of all structures that have been reported, and

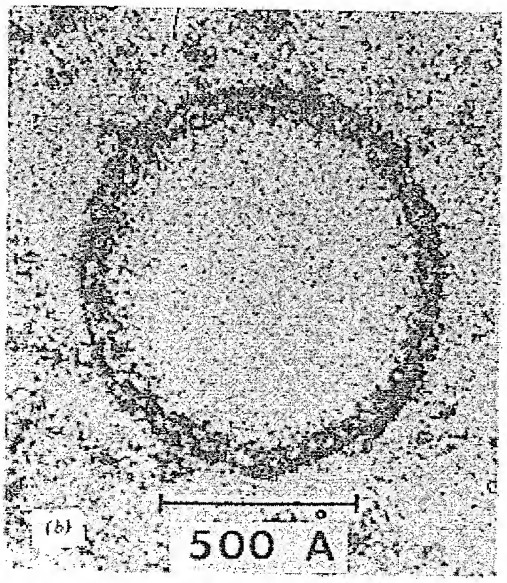


Fig. 12-1. (*continued*) (*b*) in the form of a single-walled vesicle (taken from Miyamoto and Stoeckenius, 1971).

structural studies of the ordered phases are therefore of special interest because they permit determination of bilayer dimensions.

Numerous studies have been carried out by electron microscopy (Fig. 12-1) and X-ray diffraction. The pioneering work by X-ray diffraction was done by Bear et al (1941) and Palmer and Schmitt (1941). They studied a variety of lipids from natural sources, and were able to demonstrate the existence of the bilayer structure and give essentially correct dimensions for it. In particular, they were the first to observe, in anhydrous or nearly anhydrous preparations, the spacing of 4.2 Å characteristic of hexagonally packed hydrocarbon chains perpendicular to the bilayer plane, and to show the broadening of this spacing to an average value of 4.6 Å when water was added. They correctly inferred from this that the interior of the bilayer must then be in a liquidlike state (Schmitt, 1939). More detailed X-ray studies were subsequently made by Luzzati and co-workers, and most of this work has been summarized in a review by Luzzati (1968). Phospholipids with a variety of head groups have been shown to form similar multilamellar structures (Reiss–Husson, 1967; Papahdjopoulos and Miller, 1967), including the phospholipid mixture from mitochondria (Gulik–Krzywicki et al.,

contain the electron dense acyl and phosphate ester groups). Two of these peaks are separated by about 16 Å in the dry state, and they must represent head group locations of adjacent bilayers because the separation increases to 25 Å in the wet state. The other two peaks, separated by about 43 Å in the dry state, must represent head group locations on either side of a single bilayer: the increase in their separation in the wet state occurs *outside* of the dry state peaks and represents an extension of the head groups toward the outside, with intercalation of water molecules, and does not involve an increase in the width of the hydrophobic core. The most striking aspect of the profile is the dip in the center of this region, where the electron density falls well below that of water. This dip has been interpreted as representing the location of the methyl groups at the ends of the hydrocarbon chains. Not being covalently linked, the ends of the chains approaching from the two sides would be separated by the relatively large van der Waals distance, which is more than twice the bond distance between covalently linked carbon atoms. (This fact has been previously used in the calculation of micellar volumes in Chapter 9.)

In the direction parallel to the bilayer surface only one reflection at 4.2 Å was observed, corresponding, as previously stated, to the separation between closely packed extended hydrocarbon chains. This distance did not change upon the addition of water, and the overall conclusion is that the bilayer at the temperature of measurement (23°) has a core consisting of extended hydrocarbon chains essentially perpendicular to the bilayer surface, in a regular ordered array.

Similar results were obtained with stacked bilayers consisting of egg yolk phosphatidylcholine, with the important difference that the thickness of the bilayer was found to be smaller and the surface area per head group larger: about 60 $Å^2$ per head group or 30 $Å^2$ per hydrocarbon chain. Comparison with Fig. 9-1 shows that this indicates that the hydrocarbon chains cannot on the average be fully extended in this case, as indeed would not be possible in a mixture containing unsaturated hydrocarbon chains. In addition the 4.2 Å reflection in the bilayer plane was replaced by a diffuse arc at 4.6 Å, showing not only a greater average separation between hydrocarbon chains, but also some variation from the 90° angle relative to the surface. The hydrophobic core in this case is clearly disordered, and the hydrocarbon chains must be undergoing considerable motion even though the bilayers themselves are in fixed positions. However, the trough in electron density at the center of the bilayer is still present, and the change in bilayer dimensions is not large. Substantial orientation of the chains and localization of CH_3 groups near the center evidently persists. It may be noted that increased hydration and separation between adjacent bilayers leads to slightly increased disorientation of the chains, delocalization of the ends, and slight

1967), which has a high content of cardiolipin (four hydrocarbon chains per head group). The latter finding is not surprising since the cardiolipin molecule is actually structurally equivalent to two normal phosphoglyceride molecules linked together.

The most accurate bilayer dimensions are probably those given by Levine et al. (1968) and by Levine and Wilkins (1971) who obtained highly ordered stacks of bilayers, separated by water layers, by several techniques. The advantages of such a system are obvious: discrete Bragg reflections are observed in X-ray diffraction instead of continuous diffraction envelopes, and the spacings that they represent are automatically oriented with respect to the direction of the bilayer. Although the overall resolution is relatively poor owing to the rather limited degree of order, a sufficient number of reflections were obtained in the direction perpendicular to the bilayer surface to permit analysis by Fourier synthesis to obtain an electron density profile in that direction. The phase assignment required for this procedure was based on the reasonable assumption that the changes in the repeat distance between bilayers, obtained by swelling the sample with water, are accompanied by relatively small differences in the width of an individual bilayer.

Figure 12-2 represents one repeat of the electron density profile across the stacked bilayers for dry and wet dipalmitoyl phosphatidylcholine, and the interpretation is virtually self-explanatory. The peaks of high electron density must represent the location of phospholipid head groups (which

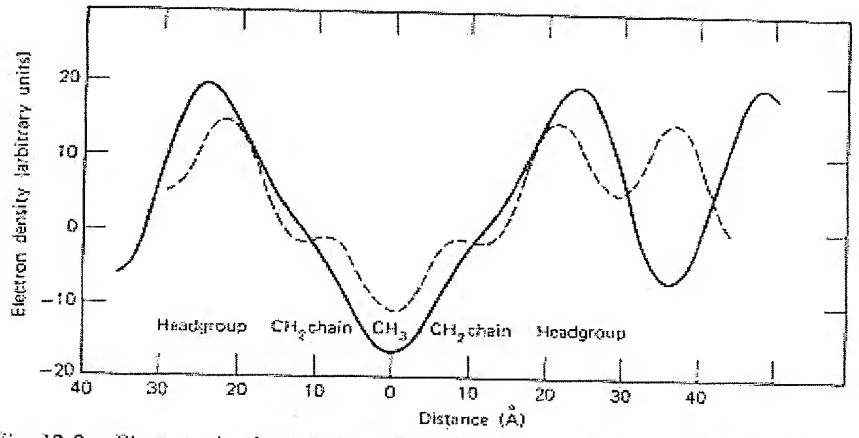


Fig. 12-2. Electron density profile of one repeat of an oriented stack of bilayers of dipalmitoyl phosphatidylcholine (taken from Levine et al., 1968). Electron densities are given relative to liquid water as zero. The dashed line represents lipid in the dry state, the solid line represents data obtained when the system is under water.

shrinkage of the core thickness. This presumably results from the greater freedom of motion of the head groups.

ARTIFICIAL MEMBRANES FOR PHYSIOLOGICAL STUDIES

For physiological studies (electrochemical properties, diffusion experiments, etc.) artificial membranes of large surface area, consisting of a single lipid bilayer, bounded on both sides by aqueous solutions, have proved to be extremely useful (e.g., Haydon, 1970). Such membranes can be prepared by a procedure first developed by Mueller et al. (1962), which involves extension and drainage of a layer of lipid in an organic solvent, such as decane, until the two monolayers at the water-organic solvent interface coalesce. Very large spherical vesicles with areas up to 1 cm^2 can be formed by closely related procedures (Pagano and Thompson, 1967). The major difference between these systems and the systems already discussed, apart from the larger surface area, is that they retain large amounts of the solvent in which the lipid was originally dissolved (Henn and Thompson, 1968). Thus, although they may serve as suitable models for a phospholipid bilayer from a physiological point of view, they cannot be considered as structurally identical.

CHOLESTEROL AND ITS INCORPORATION IN PHOSPHOLIPID BILAYERS

The state of cholesterol in aqueous solution has not been investigated, undoubtedly because its extremely low solubility makes it difficult to study. Preliminary data (Haberland and Reynolds, 1973) indicate that the monomer concentration at room temperature is limited to $10^{-8}M$, and that a micellelike aggregate exists in solution above that concentration. Because of the rigidity of the sterol ring, this aggregate may not be a micelle of the type discussed in Chapter 6, but rather an aggregate of cholesterol molecules stacked side by side. Formation of such an aggregate would not be expected to be cooperative, that is, the free energy gained by formation of a cholesterol dimer would not be expected to be significantly different from the free energy gained by adding a cholesterol molecule to an already long aggregate. Micelle formation would in that case be a process of gradual growth with increasing concentration, and would not occur as a critical phenomenon within a narrow range of total concentration. In any event the micelles or aggregates themselves have a very limited range of stability and, when the total cholesterol concentration reaches $10^{-6}M$, coalesce and separate from

the solution as a separate phase. This process is very likely due to the lack of repulsion between the hydroxyl groups that constitute the sole hydrophilic portion of the cholesterol molecule. We have already noted (page 43) that aliphatic alcohols do not form micelles, but separate as a pure liquid phase instead. Steroid derivatives with charged carboxylate groups (cholanic acids) readily form soluble micelles, although they are often of small size (Small, 1971).

Although pure cholesterol micelles in water present a considerable experimental problem, cholesterol readily enters into micelles formed by phospholipids, and mixed micelles containing as much as equimolar quantities of cholesterol and phospholipid are easily obtained and have received much attention, because the question of the biological significance of the presence of cholesterol in many membranes is an intriguing one. X-ray diffraction studies of oriented bilayers of egg yolk phosphatidylcholine containing an equimolar admixture of cholesterol (Levine and Wilkins, 1971) show that cholesterol increases the average spacing in the direction of the bilayer plane from 4.6 to 4.75 Å. The peak-to-peak distance across the bilayer is significantly increased, and there is considerable sharpening of the electron-density trough at the center of the bilayer, indicative of localization of terminal CH_3 groups over a narrower range. These results are consistent with observations on other systems (Small and Bourgès, 1966; Rand and Luzzati, 1968; Lecuyer and Dervichian, 1970) and suggest that the steroid ring of cholesterol lies in the external portion of the bilayer, as shown in Fig. 12-3,

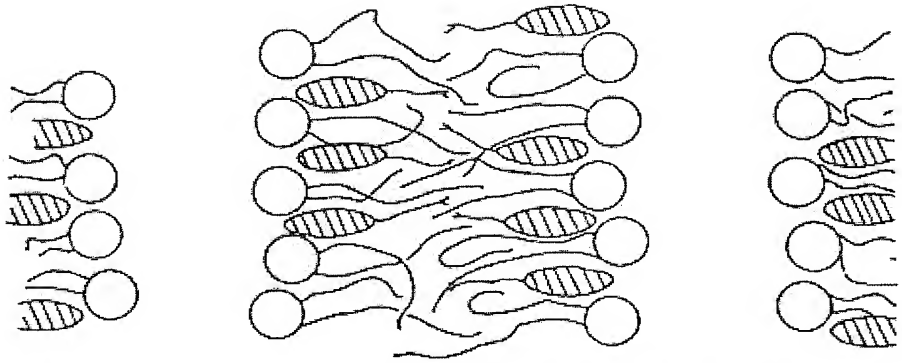


Fig. 12-3. Schematic diagram of the location of cholesterol in phospholipid bilayers (taken from Rand and Luzzati, 1968). The diagram does *not* reflect the subsequent conclusion, discussed in the text, that the phospholipid hydrocarbon chains adjacent to the cholesterol steroid rings (hatched areas) are likely to be in a rigid extended configuration, and in a much more fluid state in the center of the bilayer.

with its hydrophilic OH group in the layer occupied by the phosphatidylcholine head group, whereas the branched aliphatic tail of the molecule (see structural formula at the beginning of this chapter) lies in the central region of the bilayer. The steroid ring is not only rigid, but also has a thicker cross section than the aliphatic tail (Rothman and Engelman, 1972) and the requirement that the total area in the plane of the bilayer be the same at all levels therefore suggests that, in the external part of the bilayer containing the steroid ring, the phospholipid hydrocarbon chains will be fully extended and tend to be very close to perpendicular to the surface. The central part of the bilayer, on the other hand, will need to become very fluid, with many portions of hydrocarbon chains at a considerable angle to the perpendicular direction. The structural detail suggested by this purely geometrical reasoning will be seen to be in accordance with experimental results presented in the following chapter.

EFFECTS OF DETERGENTS

The retention of hydrocarbon in artificial membranes (see above) and the incorporation of cholesterol in phospholipid bilayers are consequences of the nonspecific nature of the hydrophobic force, and represent particular examples of phenomena already considered in a general way in Chapters 6 and 10. Any amphiphilic substance should be able to enter into phospholipid micelles, with the reservation that this could not of course occur for bilayers with an ordered core structure, such as are formed from pure phospholipids with identical saturated acyl chains, without disruption of the ordered structure.

When lysophosphatidylcholine is added to phosphatidylcholine, the bilayer structure is progressively disrupted (Bangham and Horne, 1964), and other simple amphiphiles with a single hydrocarbon chain, such as the common detergents, have the same effect. The phenomenon is again perfectly general and in accordance with the principles of mixed micelle formation set forth in Chapter 10. The equilibrium in a system of mixed amphiphiles is established regardless of the order of addition, and the addition of an excess of detergent or lysophospholipid to a system of phospholipid bilayers must progressively convert the system to one of globular detergent or lysophospholipid micelles in which small amounts of phospholipid with two hydrocarbon chains are incorporated.

It is evident therefore that detergents can be used to disrupt bilayer structures and to disperse biological lipids in soluble form within small detergent micelles. This is not of any special interest here, but it provides a rationale for the use of detergents as a tool in the investigation of biological mem-

branes: as will be shown later (Chapter 19) the lipids of biological membranes are predominantly, though not entirely, in bilayer form. Being inhomogeneous, they may often be selectively disrupted by the addition of detergents.

LIPID MONOLAYERS

Phospholipid monolayers at an air–water or hydrocarbon–water interface resemble monolayers formed by simpler amphiphiles (Chapter 11), with one important difference: because there are two hydrocarbon chains per head group, the head groups cannot approach as close to each other as in monolayers formed by amphiphiles with a single alkyl chain. Thus the minimum area per head group at high pressures is about 40 Å² instead of 20 Å². In the compression of liquidlike films interactions between head groups are similarly less important than the packing ability of the hydrocarbon chains, as is seen for example from the work of Van Deenen et al. (1962), who obtained quite similar pressure-area curves at the interface between air and 0.14 *M* phosphate buffer for the distearoyl derivatives of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and phosphatidic acid, even though the last two have negatively charged head groups. On the other hand, phospholipids containing one unsaturated and one saturated alkyl chain formed considerably more expanded films. The lack of an effect from the repulsion between charged head groups appears to be contradicted by the data of Standish and Pethica (quoted by Pethica, 1969) on films of dipalmitoyl phosphatidylethanolamine. They observed formation of condensed films at relatively low pH, where the head group is zwitterionic, but their result at pH 11.9, where the head group bears a single negative charge, resembles the curve for $C_{18}H_{37}N(CH_3)_3^+$ in Fig. 11-2.

An important observation that has been made in a number of laboratories is that cholesterol does not form liquidlike monolayers at an air–water interface, but condenses at quite low surface pressures to a solidlike film with an area of about 39 Å² per molecule (Adam, 1941, p. 49; Pethica, 1969). This result, indicating strong intermolecular forces leading to an ordered aggregate, is consistent with the results obtained for cholesterol aggregates in solution (page 106). It would be of great interest to obtain comparable data at a water-hydrocarbon interface, to determine the extent to which this specific interaction can compete with random solution of the hydrophobic portion of the cholesterol molecule in a hydrocarbon medium, but this experiment has not been done. On the basis of the ease with which cholesterol can be introduced into phospholipid bilayers (in preference to formation of pure cholesterol aggregates) one would anticipate that solidlike monolayers

would form at a water-hydrocarbon interface only at high surface pressures.

A number of investigators have reported that cholesterol exerts a condensing effect on phospholipid monolayers, that is, that the area of a mixed phospholipid–cholesterol monolayer at any pressure is smaller than would be predicted on the basis of additivity of the areas of the two components in the mixture (see review by Pethica, 1969). The significance of this observation has been questioned (Gershfeld and Pagano, 1972) because two condensed surface phases appear to coexist in at least some of these experiments.

It may be noted in conclusion that small soluble vesicles bounded by a phospholipid monolayer and containing an inner volume of an organic solvent, can be prepared by sonication of an aqueous dispersion of droplets of lipid in the organic solvent (Träuble and Grell, 1971).

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